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STUDY ON CULTURE AND ANTIMICROBIAL SUSCEPTIBILITY OF SALMONELLA TYPHI. FROM BLOOD SAMPLES

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INTRODUCTION

Some Microorganisms are pathogenic and cause infectious diseases. Microorganisms enter into the body of host either by inhalation, ingestion or injection. Once inside the body, the pathogen tries to overcome the host defensive mechanism and might get established. If it is successful the disease sets in. The physician diagnose the disease by interrogation and physical examination.

Disease caused by various members of genus Salmonella are extremely important public health problems worldwide. The credit of discovery of Salmonella goes to Salmon and Smith (1885).

The most important members of genus is Salmonella typhi, The causative agent of typhoid fever. It was first observed by Eberth(1880) and Gaffky(1884) and hence was formerly called Eberth-Gaffky bacillus or *Eberthellatyphi*.

In humans, S typhi and S paratyphi are the agents of typhoid and paratyphoid respectively, known as enteric fevers. After an incubation period they produce a septicaemia with high fever and flu like symptoms. Diarrhoea may develop but it is not the principal feature of the illness. Infection is acquired from other humans, either by direct person to person spread or indirectly via food and water contaminated by human sewage. Naturally it is a greater problem in areas of the world where sanitization is poor.

In vulnerable people, the food-poisoning salmonella can sometimes cause a typhoid like illness.

Although medical history and physical examination are the most important parts of diagnosis and therapeutic approach, they can be considered as presumptive. Correct treatment requires the confirmed diagnosis techniques. The chief function of the clinical medical laboratory are:

- To examine and culture specimens for microorganisms.
- To make accurate species identification of important isolates.
- To perform antibiotic susceptibility tests when indicated.

These tasks will assist physician in the diagnosis and treatment of infectious diseases. Microbiology data are also valuable in monitoring the course of antibiotic therapy, and in providing epidemiologic information. In addition, the laboratory also provides guidance in therapeutic management by giving the information regarding the most effective antimicrobial agent.

REVIEW OF LITERATURE

Salmonella was first visualized in 1880 by Karl Eberth in the Peyer's patches and spleens of typhoid patients. Four years later, Georg Theodor Gaffky was able to successfully grow the pathogen in pure culture. A year after that, medical research scientist Theobald Smith discovered what would be later known as Salmonella enterica. At the time, Smith was working as a research laboratory assistant in the Veterinary Division of the United States Department of Agriculture. The division was under the administration of Daniel Elmer Salmon, a veterinary pathologist. Initially, Salmonella Choleraesuis was thought to be the causative agent of hog cholera, so Salmon and Smith named it "Hog-cholerabacillus". The name Salmonella was not used until 1900, when Joseph Leon Lignières proposed that the pathogen discovered by Salmon's group be called Salmonella in his honor.

Salmonella is a genus of rod-shaped (bacillus) Gram-negative bacteria of the family Enterobacteriaceae. The two species of Salmonella are Salmonella enterica and Salmonella bongori. S. enterica is the type species and is further divided into six subspecies that include over 2,600 serotypes. (Michael Ford, 2010) Salmonella was named after Daniel Elmer Salmon (1850–1914), an American veterinary surgeon.

Salmonella species are non-spore-forming, predominantly motile enterobacteria with cell diameters between about 0.7 and 1.5 µm, lengths from 2 to 5 µm, and peritrichous flagella (all around the cell body). They are chemotrophs, obtaining their energy from oxidation and reduction reactions using organic sources. (Michael Ford 2010) They are also facultative anaerobes, capable of generating ATP with oxygen ("aerobically") when it is available, or when oxygen is not available, using other electron acceptors or fermentation ("anaerobically").

Salmonella species are intracellular pathogens; certain serotypes causing illness. Nontyphoidal serotypes can be transferred from animal-to-human and from human-to-human. They usually invade only the gastrointestinal tract and cause salmonellosis, the symptoms of which can be resolved without antibiotics. However, in sub-Saharan Africa, nontyphoidal Salmonella can be invasive and cause paratyphoid fever, which requires immediate treatment with antibiotics. (Bennett. J.E. 2008) Typhoidalserotypes can only be transferred from human-

to-human, and can cause food-borne infection, typhoid fever, and paratyphoid fever. Typhoid fever is caused by Salmonella invading the bloodstream (the typhoidal form), or in addition spreads throughout the body, invades organs, and secretes endotoxins (the septic form. (Michael Ford, 2010) This can lead to life-threatening hypovolemic shock and septic shock, and requires intensive care including antibiotics.

Habitat:

Salmonellae are associated with the intestinal intestinal tract of humans and other vertebrate animals including pigs, cows, sheep, hens, ducks and other poultry birds. (Rakesh patel and Kiran patel, 2008) Salmonellae ae excreted in the feces and urine of infected patients and are present in gall bladders of long term carriers. Salmonellae are also present in water contaminated with feces as well as in contaminated foods. Salmonellae are not killed by drying and therefore survive in products such as dried eggs.

Host Adaption:

S. enterica, through some of its serotypes such as Typhimurium and Enteriditis, shows signs of the ability to infect several different mammalian host species, while other serotypes such as Typhi seem to be restricted to only a few hosts. Some of the ways that Salmonella serotypes have adapted to their hosts include loss of genetic material and mutation. In more complex mammalian species, immune systems, which include pathogen specific immune responses, target serovars of Salmonella through binding of antibodies to structures such as flagella. (Michael Ford, 2010) Through the loss of the genetic material that codes for a flagellum to form, Salmonella can evade a host's immune system. mgtC leader RNA from bacteria virulence gene (mgtCBR operon) decreases flagellin production during infection by directly base pairing with mRNAs of the fljB gene encoding flagellin and promotes degradation. In the study by Kisela et al., more pathogenic serovars of S. enterica were found to have certain adhesins in common that have developed out of convergent evolution. This means that, as these strains of Salmonella have been exposed to similar conditions such as immune systems, similar structures evolved separately to negate these similar, more advanced defenses in hosts. Still, many questions remain about the way that Salmonella has evolved into so many different types, but Salmonella may have evolved through several phases. (Baumler et al., 2006) Salmonella most likely evolved through horizontal gene transfer, formation of new serovars due to additional pathogenicity islands. and an approximation of its ancestry. So, Salmonella could have evolved into its many different serotypes through gaining genetic information from different pathogenic bacteria. The presence of several pathogenicity islands in the genome of different serotypes has lent credence to this theory.

Salmonella sv. Newport has signs of adaptation to a plant colonization lifestyle, which may play a role in its disproportionate association with foodborne illness linked to produce. A variety of functions selected for during sv. Newport persistence in tomatoes have been reported to be similar to those selected for in sv. Typhimurium from animal hosts. (Miller, S and Pegues, 2005) The papA gene, which is unique to sv. Newport, contributes to the strain's fitness in tomatoes, and has homologs in genomes of other Enterobacteriaceae that are able to colonize plant and animal hosts.

Growth and Metabolism:

Nutrient agar: Salmonellae are facultative anaerobic and grows easily on simple nutrient media, forming moderately large (2-3 mm), round, low convex, moist, and semi-transparent colonies with entire edge. Many strains of S.paratyphi B form large mucoid colonies, the mucoid nature is due to the formation of loose polysaccharide slime.(Rakesh patel and Kiran patel, 2008)

MacConkey agar: After 18-24 hour at 37°C, lactose nonfermenting pale yellow or nearly colorless colonies are formed on MacConkey agar. (Rakesh patel and Kiran patel, 2008)

Biochemical characteristics: Carbohydrates ae general fermented with production of acid and gas. S. typhi and other anaerogenic variants form only acid. Typically glucose, mannitol, arabinose, maltose etc. are fermented, but not lactose, and sucrose. Most species of salmonella produce hydrogen sulphide. Indole not produced. Methyl-red positive. Acetylmethylcarbinol not produced. Citrate is utilized, except by S. typhi and S. paratyphi A. Gelatin is not liquefied. (Rakesh patel and Kiran patel, 2008)

Enrichment media: These are liquid media usd to assist the isolation of salmonellae from faeces, sewage, foodstuff and other material containing a mixed bacterial flora. A larger amount of the material can be inoculated into an enrichment medium than on to an agar plate, thereby facilitating the isolation of salmonellae.

Salmonellae can resist certain chemicals, which are inhibitory to coliforms; on the basis of this selective inhibition several selective and enrichment media have been formulated for the recovery of salmonellae from clinical samples. (Rakesh patel and Kiran patel, 2008)

Antigenic Structure:

Kauffmann-White classification scheme, first developed in 1934, is generally used in the clinical laboratories. Salmonellae possess three important antigens on their cell wall, based on which they are classified. (Apurba s sastry and Sandhya bhat, 2019)

The antigens are:

1. Somatic antigen (O)
2. Flagellar antigen (H)
3. Surface envelope antigen (Vi)- Found in some species.

Fimbrial antigens maybe present in some strains. They are nonspecific, wide spread among other members of Enterobacteriaceae and may cause confusion in identification.

- **Somatic antigen (O antigen)**

1. It Is a part of cell wall lipopolysaccharide(LPS)

2. Heat and Alcohol stable
3. Formaldehyde labile
4. In Widal test, O antigen of S.Typhi is used.
5. O Ag is less immunogenic
6. O antibody appears early, disappears early: indicates recent infection
7. When O antigen reacts with O antibody forms compact, granula, chalky clumps.
   - Agglutination takes place slowly.
   - Optimum temperature for agglutination is 55°C.
8. Serogrouping of salmonellae is based on the o antigen.
9. O antigen is also called the Boivin antigen because it can be extracted from the bacterial cell by treatment with trichloraacetic acid- this property was first shown by Boivin. (Apurba s sastry and Sandhya bhat, 2019)

- **Flagellar antigen (H antigen)**

1. Made up of proteins flagellin.
2. It confers motility to bacteria.
3. Heat labile and Alcohol labile.
4. Formaldehyde stable.
5. In widal test, H antigens of S. Typhi, S. Paratyphi A and B are used.
6. H antigen is more Immunogenic.
7. H antibody appears late: indicates convalescent stage.
8. When H antigen reacts with H antibody forms large, loose, fluffy clumps.
   - Agglutination takes place rapidly.
   - Optimum temperature for agglutination is 37°C.
9. Serogroups are differentiated into serotypes based on h antigens.
10. Flagellar antigen exist in two alternative phases- Phase I and II
11. Most of them are biphasic except S.Typhi which is monophasic. (Apurba s sastry and Sandhya bhat, 2019)

- **Surface envelope antigen (Vi)**

Vi antigen is a surface polysaccharide envelope or capsular antigen covering the O antigen. The naming is due to the belief that Vi antigen is related to virulence.

1. It is expressed in only few serotypes, such as S. Typhi, S. Paratyphi C, S. Dublin and some strains of citrobacter.
2. When Vi antigen is present, it renders the bacilli inagglutinable with O antiserum. However, the strain become agglutinable after boiling or heating at 100 ° C for 1 hour, which removes Vi antigen and exposes the O antigen. Vi antigen is also destroyed by 1 N HCl and 0.5 N NaOH, but not by alcohol or 0.2% formaldehyde.
3. As Vi antigen is poorly immunogenic and antibody titers are low, it is not helpful in the diagnosis of cases. Hence the Vi antigen is not employed in widal test.
4. However, it is believed that the complete absence of the Vi antibody in a proven case of typhoid fever indicates poor prognosis.
5. The Vi antibody usually disappears early in convalescence, but if persists, indicates the development of the carrier style.
6. Phage typing of S. Typhi can be done by using Vi specific bacteriophages.
7. Vi antigens can also be used for vaccination. (Apurba s sastry and Sandhya bhat, 2019)

Antigenic Variations:

The antigens of salmonellae can undergo several types of phenotypic and genotypic variations.

Variation in O Antigen:

- S-R variation is due to loss of the O antigen side chain from LPS, leading to exposure of core polysaccharide portion of LPS.
  - Smooth colonies are produced by virulent strains carrying the O antigen.
  - Rough strains from large, rough, and irregular colonies and are avirulent due to loss of O antigen. Colonies are autoagglutinable in saline suspension, and lack O serotypes specifically, hence are not suitable for antisera testing.
- Lysogenic Conversion: Infection which a bacteriophage of Salmonellae may cause loss or gain or change of an O antigen. S. Anatum is converted into S. Newington by infection with one phage and the latter into S. Minneapolis by another phage infection. (Apurba s sastry and Sandhya bhat, 2019)

Variation in H antigen:

- OH-O variation: It is associated with the loss of flagella which can be induced by:
  - Phenol agar: Growing the cultures on agar containing phenol causes inhibition of flagella temporarily which can be regained by sub culturing on the media without phenol.
  - By mutation: This is seen in non- motile mutants of S. Typhi 901-O strain, employed in the widal test. Here also, the loss of flagella is not total. The flagellated cells which are found in small numbers in such cultures can be reviewed by subculturing the culture in Craigie's tube. Alternatively, a U-tube containing soft agar may be used. (Apurba s sastry and Sandhya bhat, 2019)

- **Phase Variation:** The flagellar antigens exist in two phases. Each phase comprises of a distinct set of flagellar antigens.
  - Phase 1 antigens are serotype specific and designated as a, b, c, etc. phase 2 antigens are nonspecific or group antigens. They are few in number and are designated as 1,2, etc.

- Serotypes can be classified as:
  - Diphasic: Most salmonellae possess antigens of both phases.
  - Monophasic: Some serotypes possess only phase 1 antigens, e.g. S. Typhi, S. Agona, S. Dublin, and S. Senftenberg.
  - Aphasic: S. Gallinarum does not have any flagellar antigens. Hence, it is non- motile.

- For Seroyping, it is essential to identify the antigens of both the phases.
- Though flagger genes for antigens of both phases are present in cultures of diphasic strains, generally only one phase antigen is expressed and gets agglutinated by its phase antisera.
- Hence phase conversion has to be done to express the other phase antigens.

Phase conversion- a culture in phase 1 can be converted to phase 2 by passing it through a craigie's tube containing specific phase 1 antiserum. (Apurba s sastry and Sandhya bhat, 2019)

Pathogenesis:

Initiationofinfection: Salmonella infection begins by ingestion of sufficient number of organisms to overcome the body's defences, in particular gastric acidity, and to colonize the small intestine. Once the bacteria enter the lumen of intestine they are able to multiply. Some of the bacteria attach to microvilli of ileal mucosa by means of adhesins on the bacterial surface, which adhere specifically to mannose-containing receptors on the epithelium. Attachment is followed by degeneration of the microvilli to form breaches in the cell membrane through which salmonellae enter the cells. Further multiplication these cells and in macrophages of the Peyer's patches follows.

Although Salmonellae can cause a wide spectrum of clinical illness there are four major syndromes; these are **Enteric fever, Gastroenteritis, Bacteraemia** with or without metastatic infection and asymptomatic carrier state. (Rakesh patel and Kiran patel, 2008)

Enteric Fever:

Usually is caused by S. typhi or S. paratyphi A, B or C. The clinical features tends to be more sever with S. typhi.

After penetration of ileal mucosa organisms pass via lymphatics to mesenteric lymph nodes, where after a period of multiplication they invade the blood stream via thoracic duct. The liver, gall bladder, spleen, kidney and bone marrow become infected during this primary bacteramic phase in first 7-10 days of incubation period. After multiplication in these organs the organisms pass into the blood, causing a second and heavier bacteraemia, the onset of which approximately coincides with that of the fever and other signs of clinical illness. (Boore AL, et.al2015) From the gallbladder a further invasion with that of fever and other signs of clinical illness. From the gallbladder a further invasion of the intestine results. Peyer's patches and other gut lymphoid tissues become involved in an inflammatory reaction and infiltration with mononuclear cells, followed by necrosis, sloughing and formation of characteristic thyphoid ulcers occurs. (Rakesh patel and Kiran patel, 2008)

Onset: The interval between ingestion of the organisms and the onset of illness varies with the size of the infecting dose. It can be as short as 3 days or as long as 50 days, but is usually about 2 weeks. The onset is usually insidious. Early symptoms are often vague: a dry cough and epistaxis associated with malaise, anorexia, a dull continuous headache, abdominal tenderness and discomfort are among the most common symptoms. Diarrhea is not commonly a feature of enteric fever and early illness many patients will complain constipation. (Rakesh patel and Kiran patel, 2008)

Progression: In the untreated case the temperature shows a stepladder rise over the 1st week of the illness, remains high for 7-10 days and then decrease in a gradual step wise fashion resembling onset in reverse during 3rd or 4th week. Physical signs include relative bradycardia for the height of fever, hepatomegaly, splenomegaly and often maculopapular rash- 'rose spots'. These are 2-4 mm in diameter, slightly raised discrete irregular blanching pink macules most often found in the front of the chest and abdomen. They appear in crop of up to a dozen at a time and fade after 3 or 4 days, leaving no scar. (Rakesh patel and Kiran patel, 2008)

If illness is not treated, the fever and the other findings usually continue for 3-4 weeks, then in the preantibiotic era, these complications were responsible for mortality as high as 20% in typhoid fever. With adequate antimicrobial therapy the infection, although serious, is fatal in less than 1% of cases.

Paratyphoid fever have similar symptoms but is milder than typhoid caused by S. typhi. (Rakesh patel and Kiran patel, 2008)

Gastroenteritis:

This is the commonest manifestation of salmonella infection caused by S. enteritidis and S. typhimurium. Salmonellosis has an incubation time of 8-48 hours. The salmonellae first invade the instestinal mucosa and multiply there. Sometimes they manage to pass through the instestinal mucosa and multiply there. Sometimes they manage to pass through the instestinal mucosa to enter lymphatic and cardiovascular systems, and from there they may spread to eventually affect many organs. (Cremon C, et.al, 2014) The fever associated with Salmonella infection might be from endotoxins released by lysed cells.

Illness begins with nausea, vomiting, and diarrhea of varying severity 48 hours after ingestion. Myalgia, headache, fever and other constitutional symptoms may also develop. The diarrhea is usually intermediate in nature between the voluminous watery diarrhea of cholera and crampy diarrhea of shigellosis. Gross blood and pus are not common, but red cell and white cells may be seen on microscopic examination of stool. In more severe cases, dehydration leading to hypotension, cramps and renal failure is observed. Vomiting is rarely a prominent feature of the illness. Infection tends to be more common, sever and prolonged in infants, the aged and the sick than in healthy adults (Porter CK, et.al,2013) The usual case is self-limiting without extension beyond gastrointestinal tract.

Septicemia:

Bacteraemia is a constant feature of enteric fever. Occasionally the wide spread dissemination of organism throughout the body results in the establishment of one or more localized foci of persisting infection, especially where preexisting abnormality makes a tissue or organ vulnerable. Artherosclerotic plaques

within large arteries, damaged heart valves, joint prostheses and other implants are all susceptible to metastatic infection.

People with sickle cell anemia are at high risk of developing osteomyelitis. Salmonella meningitis is particularly serious complication of infection in neonates & very young children.

Abscess formation can occur in almost any organ or tissue. Even in absence of obvious tissue damage the ability of salmonellae to enter and survive within macrophages and other cells, particularly in the liver and biliary tree, but also in bone marrow and kidney, occasionally leads to persistent infection and chronic carrier state. (Rakesh patel and Kiran patel, 2008)

Epidemiology:

Host: Humans are the only natural hosts for typhoidal salmonellae. (Apurba s sastry and Sandhya bhat, 2019)

Mode of transmission: It is by ingestion of contaminated water and food. Rarely homosexual and laboratory acquired transmission have been reported. (Apurba s sastry and Sandhya bhat, 2019)

Prevalence: worldwide, an estimated 27million cases of enteric fever with 2-6 lakh deaths occur annually. (Apurba s sastry and Sandhya bhat, 2019)

Incidence is:
- Highest (>100 cases per 1000,000 population per year) in south central and southeast Asia.
- Medium (10-100 cases per 100,000) in rest of Asia, Africa, Latin America.
- Low (<10 cases per 1000,000) in other parts of the world. (Apurba s sastry and Sandhya bhat, 2019)

Locality and age: Enteric fever is:
- More common in urban than rural areas.
- More common among young children and adolescents than in adults. (Apurba s sastry and Sandhya bhat, 2019)

Factors that favor transmission include:
- Poor sanitization and improper cleaning of drinking water.
- Contaminated water, food and drinks.
- Lack of hand washing and toilet access, and evidence of prior Helicobacter pylori infection (Kass EH, 1987)

Typhi vs Paratyphi: S.Typhi infection is more common than S.Paratyphi A (ratio is 4:1). However, S.Paratyphi A appears to be increasing especially in India; maybe due to increased vaccination for S.Typhi. (Apurba s sastry and Sandhya bhat,2019)

Carriage: Up to 10% of untreated patient become carriers and excrete S.Typhi in feces or urine.
- Carriers are of two types:
- Fecal carriers: Salmonellae are associated with intestinal tract of humans & other animals. Typhoid Bacilli multiply in the gall bladder and are excreted in feces. Fecal carriers are more common.
- Urinary carriers: Multiplication takes place in kidneys and bacilli are excreted in urine. Urinary carriers are rare.
- Duration of shedding: Carriers continue to shed the bacilli in feces and urine for:
- Convalescent carriers 3 weeks to 3 months (after clinical cure).
- Temporary carriers 3 months to 1 year.
- Chronic carriers for more than 1 year.(Besser JM, 2018)
- **Chronic carriers** occur in about 1-4% of infected people. Chronic carriage is more common in:
- Women, Infants and old age.
- Biliary tract abnormalities which leads to increased fecal excretion
- Abnormalities of urinary tract and associated Schistosomahaematobium infection of bladder leads to increased urinary excretion. (Apurba s sastry and Sandhya bhat, 2019)
- **Food handlers or cooks** who become chronic carriers are particularly dangerous, can excrete the bacilli for many years. The best known example of such typhoid carrier was Mary Mallon ("Typhoid Mary"), a New York cook who gave rise to more than 1300 cases during her lifetime causing several outbreaks. (Apurba s sastry and Sandhya bhat, 2019)
- **Reference centers** for Salmonella in India:
- National Salmonella Phage Typing center- at Lady Hardinge medical college, New Delhi.
- National Salmonella Reference center- at central Research Institute, Kasauli.
- National Salmonella Reference Centre for animal origin- at Izatnagar. (Apurba s sastry and Sandhya bhat, 2019)

MATERIALS AND METHODS

The present study was conducted at Department of Microbiology, Green Cross Pathology and Molecular Laboratory Ahmedabad during the period of 1st January 2021 to 1st February 2021. All Salmonella strains (non-repetitive) isolated from blood samples received at department of Microbiology during the study period were included in study.

BacT/Alert method:

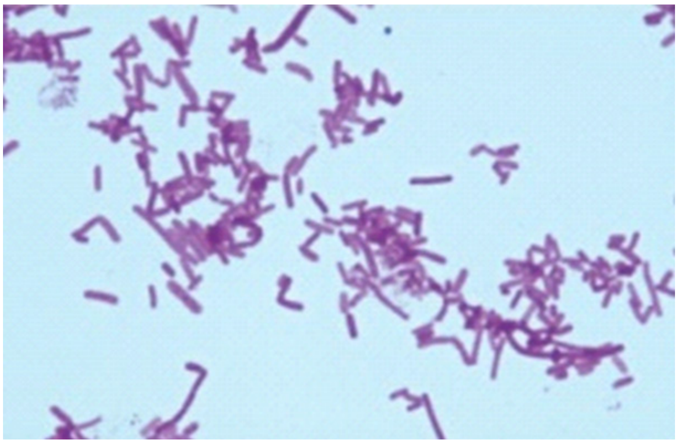
BacT/Alert is based on colorimetric detection of growth.
- BacT/ Alert bottles contain Tryptic soy broth and brain heart infusion broth added with adsorbent polymeric beads which neutralize the antimicrobials present in blood specimen.
- A sensor (liquid emulsion sensor) is bonded to the bottom of each bottle and separated from broth medium by a differentially permeable membrane.
- Co2 produced by growing microorganisms diffuses across the membrane into the sensor where it reacts with water generating hydrogen ions.
- As the concentration of hydrogen ions increases and the pH decreases, the blue-green sensor becomes yellow, a change that is detected by colorimetric method.
- The algorithm for detection of growth is based on an analysis of the rate of change of Co2 concentration occurring in each individual bottle.

Procedure for Dealing with negative bottles:
The majority of blood culture bottles received in the laboratory shows no growth and should be discarded after 5-7 day's incubation. If a continuous monitoring system is used a terminal subculture need not to be carried out. However if a traditional manual blood culture system is used it is common for a blind sub culture to be carried out. There are some exceptions where bottle may require long incubation before discarded, for example endocarditis or in specific patients where there is a possibility of an infection with a fastidious slow growing organism. For negative bottles a written report is issued.
Procedure for dealing with positive bottles:

There are three stages in this process:
- Microscopy
- Culture and sensitivity tests
- Identification of the pathogen.

Microscopy
The first stage in processing is to carry out a gram stain on the blood culture:
- The aim of a Gram stain of a positive blood culture bottle is to provide the clinician with a guide to the identity of a possible pathogen, allowing initiation of appropriate antibiotics. Up to 24hours may elapse before susceptibility test results and the organism's identity are known; initial therapy is guided by the Gram stain result.
- A presumptive identification of the bacteria in a positive blood culture is invaluable to clinicians in assessing the requirement for and type of antimicrobial therapy appropriate for a patient.

Procedure:
- A few drops of blood are smeared onto a glass microscope slide, air dried and heat fixed.
- The slide is flooded with crystal violet stain, which is allowed to set for 30-60 seconds.
- The crystal violet is rinsed off with gentle stream of water.
- The slide is flooded with gram's iodine, which is allowed to set for 60 seconds.
- The iodine is removed with gentle stream of water.
- The slide is decolorized by rinsing with 95% ethanol-acetone, drop by drop, until it rinses clear.
- The slide is flooded with saffranine, which is allowed to set for 30 seconds.
- The saffranine is rinsed off with stream of water.
- The excess water is removed by blotting with bibulous paper.
- The slide is allowed to air dry.
- The slide is observed under the microscope using both low power and oil immersion lenses.



Culture and Sensitivity Tests.
Culture Methods:
The Bacteriological culture is done in a laboratory, for the following purpose:
- Isolating bacteria in pure culture from the clinical specimens.
- To perform biochemical tests for identification of bacteria.
- To perform antimicrobial susceptibility testing of the isolated bacteria.
- To obtain sufficient growth for the preparation of antigen.
- For typing of bacterial isolates.
- To estimate the viable bacterial count.

Streak culture:
A loopful of specimen is smeared onto the surface of a dried solid culture plate near the peripheral area with the help of a sterile bacteriological loop to form the primary inoculum.
- From the primary inoculum, it is spread thinly over the culture plate by streaking with the loop in parallel lines to form the secondary, tertiary inoculum and finally a feathery tail end.
- Intermittent heating: The loop is flamed and cooled in between the different set of streaks to get isolated colonies.
- The culture plate is incubated at 37°C for 12-18 hours (overnight).
- Confluent growth occurs at the primary inoculum and isolated colonies are obtained on the final streaks.
- Obtaining isolated colonies is a prerequisite to perform various biochemical tests to confirm the identification of bacteria.

Antibiotic Susceptibilty Testing:
Principle:
The antibiotic impregnated disc absorbs moisture from the agar and antibiotics diffuses into the agar medium. The rate of extraction of the antibiotic from disc is greater than the rate of diffusion as the distance from the disc increases, there is a logarithmic reduction in the antibiotic concentration. Visible growth of bacteria occurs on the surface of the agar where the concentration of antibiotic has fallen below its inhibitory level for the test strain. The point at which the critical cell mass is reached appears as a circle of bacterial growth, with the middle of the antibiotic disc forming the center of the circle.

Procedure:
- Prepare plates with Muller Hinton agar for use in the Kirby Bauer method for rapidly growing aerobic bacteria. The medium in the plates should be sterile and have a depth of about 4mm.
- Use pure culture as inoculum. Select 3-4 colonies and transfer them into about 5ml of suitable broth such as Peptone Broth. Incubate at 37°C for 2-3 hours.
- Dip a sterile non-toxic cotton swab in the previously incubated peptone broth. Streak the entire agar surface of the plate with the swab three times, turning the plate at 60° angle between each streaking. Allow the inoculum to dry for 5-15 minutes with lid in place.
- Apply the discs using aseptic technique. Deposit the discs with centers at least 24mm apart. Incubate immediately at 37°C and examine after 18-24 hours.
- Measure the zones showing complete inhibition and record the diameter of the zones to the nearest millimeter. (Known Sensitive control must be used.)

Identification of the pathogen:
Identification of pathogen majorly done by two types of methods:
- Biochemical Reactions.
- Automated system used for bacterial identification (VITEK 2).

Biochemical reactions.
Based on the type of organisms observed in culture smear, the appropriate biochemical tests are employed.

For gram-negative bacilli:
- Citrate utilization Test.
- Urea Hydrolysis Test.
- Triple Sugar Iron Agar Test.
- Phenyl Pyruvic Acid Test.
- Methyl Red (MR) Test.
- Motility Test.
- Indole Test.

- **Citrate utilization Test.**

It detects the ability of a few bacteria to utilize citrate as the sole source of carbon for their growth, with production of alkaline metabolic products. Citrate test is performed on a citrate containing medium, such as Simmon's citrate agar.

Simmon's citrate medium: Citrate utilizing bacteria produce growth and a color change, i.e. original green color changes to blue.

- **Urea Hydrolysis Test.**

Urease producing bacteria can split urea present in the medium to produce ammonia that makes the medium alkaline.

Test is done on Christensen's urea medium, which contains phenol red indicator that changes to pink color in alkaline medium.

- **Triple Sugar Iron Agar Test.**

Composition:
It is a composite solid agar medium in tube having a butt and a slant. Its constituents include:
- Three sugars- Glucose, Sucrose and lactose in the ratio of 1:10:10 parts
- Phenol red as an indicator of acid production.
- Ferric salts as an indicator of hydrogen sulfide production.

Procedure:
Medium is inoculated with a pure bacterial culture by a straight wire pierced deep in the butt (stab culture) and then doing a stroke culture on the slant area. The tube is incubated at 37 °C for 18-24 hours. Under incubation or over incubation may lead to false interpretation of result.

Interpretation:
TSI detects three properties of bacteria, such as fermentation of sugars to produce acid and/or gas and production of H2S.
- **Ability to ferment sugars to produce acid:** Uninoculated TSI medium is red in color and on acid production the color changes to yellow. Based on fermentation of sugar presents in TSI, the organisms are categorized into three groups.
- **Nonfermenters:** They do not ferment any sugars, hence an alkaline slant and alkaline butt reaction is observed.
- **Glucose only fermenters:** They ferment only glucose and produce little acid. Initially at 8 hours, the whole medium turns acidic. Later on, the organism begins oxidative degradation of the peptones present in the slant, resulting in alkaline by-products in slant, which change the indicator back to red color.
- **All sugars fermenters:** They ferment glucose and also ferment lactose and/or sucrose to produce large amount of acid so that the medium turns acidic at 8 hours.
- **Ability to produce gas:** Some bacteria produce gas by sugar fermentation; which is denoted by breaks/ cracks in the medium or the medium is lifted up.
- **Ability to produce H2S:** Certain bacteria produce hydrogen sulfide, which is a colorless gas. H2S combines with ferric ions to form ferrous sulfide, that produces blackening of the medium.

Phenyl Pyruvic Acid Test:
This is a specific test done for members of tribe proteeae; which includes Proteus, Morganella and Providencia. They possess a specific enzyme that deaminates phenylalanine present in the medium to phenyl pyruvic acid. PPA reacts with few drops of 10% ferric chloride solution to produce green color.

Methyl Red (MR) Test:
In glucose phosphate broth, certain bacteria ferment glucose to produce stronger acids that maintain the pH below 4.4, which turns methyl red indicator from yellow to red color.

Motility Test:
Motility testing using a semi-solid medium is a commonly used for the identification of gram-negative bacteria of Enterobacteriaceae family. Motility testing is done in

conjugation with other biochemical testing using special biochemical media such as Mannitol agar medium.

Procedure:
- Prepare a semisolid agar medium in a test tube.
- Inoculate with a straight wire, making a single stab down the center of the tube to about half the depth of the medium.
- Incubate at 37 °C.
- Examine at intervals.

Results: Hold the tube up to the light and look at the stab line to determine motility.
- Non-motile bacteria: generally gives growth that are confined to the stab line, have sharply defined margins and leave the surrounding medium clearly transparent.
- Motile Bacteria: typically gives diffuse, hazy growth that spread throughout the medium rendering it slightly opaque.

Indole Test

This test is performed on peptone broth medium inoculated with bacterial species to determine the ability of the organism to convert tryptophan into indole. Formation of red colored ring indicates a positive result whereas formation of yellow colored ring indicates a negative one.

Automated system used for bacterial identification (VITEK 2)

The VITEK 2 is an automated system used for identification and antimicrobial susceptibility testing of bacteria and yeast.

It uses colorimetric reagent card containing 64 wells; each well contains an individual test substrate. Separate cards are available for gram-negative, gram-positive bacteria, fastidious bacteria and yeast.

- **Identification:** Substrates in the well measure various metabolic activities such as acidification, alkalinization, enzyme hydrolysis, etc. which helps in identification of the organism. The reaction pattern obtained from the test organism is compared with the database and the identification is reported with confidence level of matching.
- **Antimicrobial susceptibility test:** It works on the principle of microbroth dilution. The wells in the card contain doubling dilution of antimicrobial agents. The organism suspension is added to the wells. The minimum inhibitory concentration (MIC) is determined as the highest dilution of the antimicrobial agent which inhibits the growth of organism.
- **Incubation:** The cards are incubated in the system at 35.5± 1°C. The reading is taken once every 15 minutes by the optical system of the equipment which measures presence of any colored products of substrate metabolism or turbidity for AST.

The results of identification is usually available within 4-6 hours and AST within 16-18 hours.

RESULTS

The present study carried out during 1st January 2021 to 1st February. Total 12 numbers of samples were analyzed.

Salmonella Typhi Infection Ratio in Men and Women

Sex	Total	Percentage
Female	6	50%
Male	6	50%

Salmonella Typhi Infection Ratio in Different Age

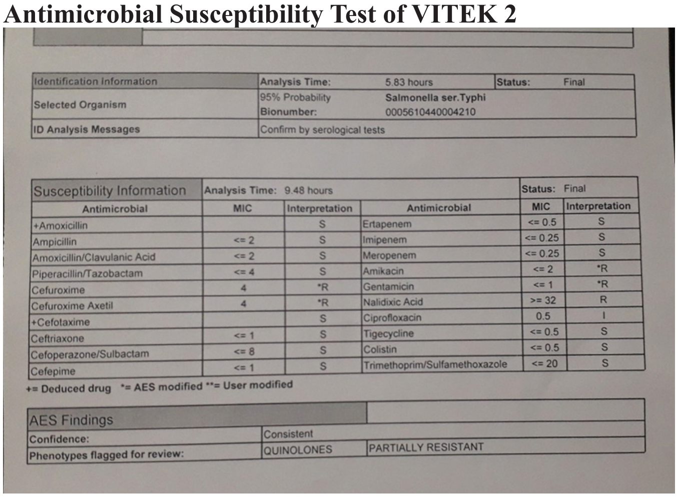
Age	No. of infection	Percentage
0-5	9	75%
5-10	1	8.33%
10-15	2	16.66%

List of Antibiotic Drugs

Antimicrobial Drug	Resistant	Intermediate	Sensitive
Aminoglycosides			
Amikacin	100%	0.0%	0.0%
Gentamicin	100%	0.0%	0.0%
Netimicin	100%	0.0%	0.0%
Tobramycin	100%	0.0%	0.0%
β-lactam/penicillins			
Amplicillin	0.0%	0.0%	100%
Amoxycillin	0.0%	0.0%	100%
Piperacillin	0.0%	0.0%	100%
β-lactam/ β-lactamase inhibitor combinations			
Amoxycillin/ Clavulanic Acid	0.0%	0.0%	100%
Ampicillin/ Sulbactam	0.0%	0.0%	100%
Piperacillin/ Tazobactam	0.0%	0.0%	100%
Ticarcillin/ Clavulanic acid	0.0%	0.0%	100%
Cefoperazone/ Sulbactam	0.0%	0.0%	100%
Cefepime/ Tazobactam	0.0%	0.0%	100%
Carbapenems			
Imipenem	0.0%	0.0%	100%
Meropenam	0.0%	0.0%	100%
Ertapenem	0.0%	0.0%	0.0%
Cephalosporins (1st& 2nd generation)			
Cephalothin	100%	0.0%	0.0%
Cefazolin	100%	0.0%	0.0%
Cephalexin	100%	0.0%	0.0%
Cefadroxil	100%	0.0%	0.0%
Cefaclor	100%	0.0%	0.0%
Cefuroximel (Parenteral)	100%	0.0%	0.0%
Cephalosporins (3rd& 4th generation)			

Cefixime	0.0%	0.0%	100%
Cefoperazone	0.0%	0.0%	100%
Cefotaxime	0.0%	0.0%	100%
Ceftazidime	0.0%	0.0%	100%
Ceftriaxone	0.0%	0.0%	100%
Cefitizoxime	0.0%	0.0%	100%
Cefepime	0.0%	0.0%	100%
Cefepirome	0.0%	0.0%	100%
Tetracycline			
Azithromycin	0.0%	0.0%	100%
Tetracyclin	0.0%	0.0%	100%
Tegycycline	0.0%	0.0%	100%
Quinolones			
Ciprofloxacin	75%	8.33%	16.66%
Moxifloxacin	0.0%	0.0%	100%
Levofloxacine	0.0%	0.0%	100%
Nalidixic acid	100%	0.0%	0.0%
Norfloxacine	0.0%	0.0%	100%
Ofloxacine	0.0%	0.0%	100%
Pefloxacine	0.0%	0.0%	100%
Sparfloxacine	0.0%	0.0%	100%
Lomefloxacine	0.0%	0.0%	100%
Gemifloxacine	0.0%	0.0%	100%
Prulifloxacine	0.0%	0.0%	100%
Others			
Chloramphenicol	0.0%	0.0%	100%
Co-trimoxazole	0.0%	0.0%	100%
Aztreonam	0.0%	0.0%	100%
Colistin	0.0%	0.0%	100%
Polymyxin B	0.0%	0.0%	100%

Antimicrobial Susceptibility Test of VITEK 2



Identification Information	Analysis Time:	5.83 hours	Status: Final
Selected Organism	95% Probability Bionumber:	Salmonella ser.Typhi 0005610440004210	
ID Analysis Messages	Confirm by serological tests		

Susceptibility Information	Analysis Time: 9.48 hours			Status: Final	
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
+Amoxicillin		S	Ertapenem	<= 0.5	S
Ampicillin	<= 2	S	Imipenem	<= 0.25	S
Amoxicillin/Clavulanic Acid	<= 2	S	Meropenem	<= 0.25	S
Piperacillin/Tazobactam	<= 4	S	Amikacin	<= 2	*R
Cefuroxime	4	*R	Gentamicin	<= 1	*R
Cefuroxime Axetil	4	*R	Nalidixic Acid	>= 32	R
+Cefotaxime		S	Ciprofloxacin	0.5	I
Ceftriaxone	<= 1	S	Tigecycline	<= 0.5	S
Cefoperazone/Sulbactam	<= 8	S	Colistin	<= 0.5	S
Cefepime	<= 1	S	Trimethoprim/Sulfamethoxazole	<= 20	S

+= Deduced drug *= AES modified **= User modified

AES Findings		
Confidence:	Consistent	
Phenotypes flagged for review:	QUINOLONES	PARTIALLY RESISTANT

DISCUSSION

The first objective of the study was to determine virulence factor produced by Salmonella typhi. among the people of Ahmedabad.

The rate of infection inrelation to gender is summerized in table 4.1. from the table it can be seen that female and male both are infected equally. During the study of 12 positive samples were isolate, total 6(50%) female and 6(50%) male were infected which shows infection ratio of Salmonela typhi. is equal in male and female.

In our present study out of 12 cases positive for Salmonella typhi.included, majority of the patients were found to be in the age group of 0-5 years (75%) as seen in table 4.2.

In the context of antibiotic sensitivity test as seen in table 4.3 antibacterial agents like β-lactam/penicillins (100%), β-lactam/ β-lactamase inhibitor combinations (100%), Carbapenems (100%), Cephalosporins (3rd& 4th generation) (100%), Tetracycline (100%), Quinolones (100%) were most effective antibiotic agent followed by Chloramphenicol(100%), Co-trimoxazole(100%), Aztrenam(100%), Colistin (100%) and Polymixin B (100%).

The sensitivity of Salmonella typhi to the Ciprofloxacin (16.66%), Aminoglycosides (0%), Cephalosporins (1st& 2nd generation) (0%), Nalidixic acid (0%).

Antimicrobial resistance is a big problem cause of great concern throughout the world. Knowledge of the antibacterial resistance among the Salmonella typhi infection is essential to provide appropriate cost effective therapy.

CONCLUSION

While screening of 12 number of samples totally we found 50% female and 50% male are infected by Salmonella typhi. From the screening blood samples and characterization of isolates we can conclude that infection is equal in female and male.

This study was carried out on varies age groups, the majority of Salmonella typhi. is isolated from 0-5 years.

The main objective of present study was to find out the drug resistance pattern of Salmonella typhi. from blood culture. We have performed antibiotic susceptibility test from various drugs.

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